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ANTIBODY TO THE RNA-DEPENDENT DNA POLYMERASE OF HTLV-III: CHARACTERIZATION AND CLINICAL ASSOCIATIONS

FINAL REPORT

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his project was designed to explore B cell epitopes of the HIV-1 polymerase which are mportant to the identification of in vitro immunologic responses correlating with progres- ion of HIVdisease, and may provide targets for immune intervention. We first reported a eterogeneous immune response for inhibition of HIVreverse transcriptase activity by IgGs rom HIVseropositive individuals. Loss of this inhibitor appeared to be associated with evelopment of clinical disease, a finding which has been confirmed by at least three groups orld-wide. We subsequently attempted to identify a linear sequence of the polymerase which ould be used in a more rapid serologic assay to define the epitope reactivity of these anti- T antibodies. A statistically significant correlation between anti-RT serologic reactivity nd RT enzymatic inhibition was found utilizing two peptides from conserved, amino-terminus esidues of the polymerase. Further epitope mapping is in progress, in concert with develop- ent of a in vivo mouse model to evaluate the efficacy of antibody reagents in concert with ell activation inhibitors to block progression of HIV infection.

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Purpose: To explore epitopes related to the HIV-1 polymerase which are important both to the identification of in vitro cellular responses correlating with disease progression, and as targets for immune intervention in HIV infection.

Relevance to WRAIR Plan: 1. Definition of viral epitopes which should serve as targets for vaccine development, passive immunization, and cytotoxic T cell priming.

2. Continued exploration of the relevance of viral activation to immune intervention.

Collaboration with WRAIR Programs: 1. Use of the large bank of serial serum samples from a population in which clinical and laboratory data are available.

2. Possibility of using our newly developed transgenic mouse model to evaluate polymerase epitopes identified in this project as targets for immune intervention.

PUBLICATIONS, ALL ACKNOWLEDGING USAMRDC SUPPORT AND RELEVANT TO THIS PROPOSAL:

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I. MAJOR PROJECT

Background: The serum of many animals naturally infected with retroviruses contain antibodies capable of blocking the enzymatic activity of particulate reverse transcriptases. Stimulated by the correlations of antibody to reverse transcriptase (RT) with clinical status first identified in cattle and cats, we reported a heterogeneous immune response for inhibition of HIV-associated RT function by IgGs from HIV-infected individuals (1). Loss of this inhibitor appeared to be assocated with development of clinical disease (1).

This finding, and the clinical associations, were subsequently confirmed by two other groups (2,3). The probability that this functional assay served to identify discrete subsets of anti-polymerase antibodies was strengthened by the fact the p66/p51 RT of HIV-1 (4,5) and HIV-2 (6) are highly immunogenic, detectable by immunoblotting in >90% of seropositive individuals, and do not correlate with clinical stages of HIV infection (4). Such serologic studies cannot, of course, detect changes in epitope specificity among antibody species as the disease progresses.

There is a need to explain why HIV RT, a core protein that is present at substantially lower concentrations in the virion than structural core proteins such as p24, is so uniquely immunogenic. Indeed, HTLV-I and HTLV-II are not commonly associated with anti-RT responses (4). Perhaps continuous cell

lysis in HIV infection constantly challenges the hosts immune system with RT. This could induce not only the strong humoral responses reported above, but also strong anti-pol cytotoxic T lymphocyte responses discovered by others (7). Longitudinal studies with this CTL anti-pol assay system are also ongoing, with preliminary reports demonstrating a correlation between loss of MHC class I restricted CD8+ T cell activity against RT targets and disease progression (7).

Present data: In an attempt to define a linear sequence of the HIV pol which could be used in a serologic assay to define these anti-RT inhibitory antibodies, synthetic peptides were prepared from nucleotide sequences of HIV pol regions homologous to the catalytic sites of other mammalian polymerases. The initial peptides, ranging in length from 8 to 15 amino acids, were synthesized at The New York Blood Center by the solid-phase method using an automated peptide synthesizer (Applied Biosystems, Foster City, CA). Peptide resins were cleaved by hydrogen fluoride, extracted, and analyzed for purity by HPLC.

The five sequences we initially employed were:

Code	Sequence	AA Position	Activity	Reference
I	LDVGDAYF	109-116	NTP binding site	8
II	VLPQGWKGSP	158-157	conserved region	8
III	IQKLVGKLNW	257-266	conserved region	8
IV	PENPYNTPVFAIKK	219-232	conserved region	9
V	DSRNPLWKGPAKLLW	496-509	COOH terminus	9

ELISA. Peptide solutions of 10mg/ml in PBS were air drived overnight at 37^{0}C in polyvinyl, 96-well microtiter plates to yield 1-5 ug/well. Nonspecific binding sites were blocked with BSA. After a 6 h incubation at 25^{0}C and a 16h incubation at 4^{0}C, serum samples were added at 1:100, 1:200 and 1:400 dilutions. After 2 h at 25^{0}C the wells were washed 10-20 times with 0.2% Tween 20 in PBS, after which affinity-purified, alkaline phosphatase conjugated goat antibody to human IgG was added. After 2 h the plates were washed, and substrate solution was added. After a 30 in. incubation the reaction was stopped by adding 1N H2S04, and the optical densities were analyzed on an automated ELISA scanner. Seropositivity was defined as any value greater than three standard deviations obtained with negative controls.

SAMPLES. The samples analyzed included 100 sera obtained from our original longitudinal survey (1979-1985). An additional 150 samples have been obtained from Dr. Robert Redfield at WRAIR and are in the process of being analyzed.

A statistically significant but imperfect correlation was found between the presence of antibodies capable of blocking the catalytic activity of RT, as reported by our functional assay (1) and anti-peptide I, II or IV reactivity. The latter two residues correspond to the putative HIV catalytic site as determined by insertional mutagenesis (8, 9). Interestingly, they are outside the regions of the pol gene thought to be immunogenic by

hydrophobicity plots (10) or B cell eiptope computer analysis (11), yet are within the few discrete regions of pol predicted to be strong T cell epitopes (11).

The data, gathered on 100 HIV seropositive individuals at various clinical stages of infection and 15 seronegative controls were as follows:

0/15 HIV seropositive individuals were positive by ELISA with any of the 5 synthetic peptides.

38/100 HIV seropositive individuals were positive for one or more pol peptides. Of these 38, only 3 had WR stage 5-6 disease, with all others in the WR 1-2 category.

Of the 62 seropositive individuals who were non-reactive, all were more advanced than WR 2, and only 6 had anti-RT catalytic activity.

The raw data for the 38 reactive patient samples were:

Peptide No.	I	II	III	IV	V
No. sera +	31	26	3	8	3
% positive	81.6	68.4	7.9	21.1	7.9

Serologic Reactivity vs. Anti-RT inhibitory Activity

	Serologic Activity to peptides II and/or IV	No serologic activity for II and/or IV
No anti-RT activity	2	7
Anti-RT activity	20	9

Interestingly, Peptides III and V gave low frequencies of positive reactions similar to those for pol synthetic peptides selected on the basis of hydrophobicity plots (10).

II. Attempts to correlate anti-RT reactivity with standard neutralization assays or p24 antigenemia.

The possibility that these anti-RT IgGs correlated with existence of other antibodies with reactivity against neutralizing envelope epitopes was investigated. This latter area is controversial. Virus neutralizing factors have been found in >50% of serum samples obtained from AIDS and ARC patients (12), and in a higher number of HIV seropositive asymptomatic carriers (13). Clinical correlations in patients followed over time has been weak, however. The assays typically involve single HIV isolates as targets, albeit envelope variability is well documented among HIV strains, even from a single individual (14).

(a) We attempted to link HIV neutralization in vitro with anti-RT activity, using IgG from of the asymptomatic carriers. Using $\geq$ 75% inhibition of enzyme activity as the criterion for neutralization, we found most samples effective at 10-100 ug IgG, without regard to the patient's clinical status or the anti-RT

capacity of the IgG.

(b) We have received 78 (38 HIV seropositive) serum samples from Dr. Zvi Bentwich, Kaplan Hospital, Rehovot, Isreal, together with clinical and immunologic data. We have correlated serum p24 antigen levels with anti-RT activity and related these to clinical course, as described below:

Group I: n = 24
HIV seropositive
Clinical data: CD4+ T cells < 400/mm3
lymphadenopathy or other clinical manifestations of HIV infection

Group II: n = 14
HIV seropositive
Clinical data: CD4+ T cells ≥ 400/mm3
asymptomatic

Group III: n = 13
HIV seronegative
Clinical data: "non-HIV immune defects"

Group IV: n = 12
HIV seronegative
Clinical data: HIV at risk group, normal immune function.

Group V: n = 15
HIV seronegative
Clinical data: non-HIV risk group, normal immune function.

These sera were evaluated for antibody against HIV-1 RT catalytic activity, as well as for circulating p24 antigen by ELISA-based antigen capture (Abbott Labs, Chicago, IL). As shown in Table I, 6/24 (25.0%) of Group I samples were positive for p24 antigenemia, while 1/14 (7.1%) of Group II samples were as well. In contrast, anti-RT antibodies were noted in 2/24 (8.3%) of Group I, but 8/14 (57.1%) of Group II samples. These data

Table I. Correlation of p24 antigenemia and anti-RT antibodies with degree of clinical or immune deficit in HIV infection.

GROUP I: HIV seropositive, symptomatic			GROUP II: HIV seropositive, asymptomatic, CD4+ T cells >400/mm3		
Sample code	p24 antigen (pg/ml)	anti-RT	Sample	p24 antigen (pg/ml)	anti-RT
778	1212	-	99	0	-
840	0	-	662	0	+
261	0	-	272	14	-
713	0	-	91	0	+
589	0	+	365	0	+
288	0	+	661	0	+
634	0	-	222	0	+
842	0	-	759	0	+
241	240	-	641	0	-
482	0	-	67	0	-
432	102	-	580	0	-
878	>8000	-	765	45	-
830	0	-	169	0	-
449	0	-	254	0	-
668	40	-			
320	0	-			
289	0	-			
815	0	-			
930	5152	-			
707	0	-			
966	0	-			
626	0	-			
469	0	-			
237	0	-			
% positive	25.0	8.3		7.1	57.1

support our original observation of association of such antibodies with improved clinical status. It also emphasizes the fact that while p24 antigenemia tends to be associated with advancing clinical stage of HIV infection, this correlation is far from perfect. Combination of anti-RT antibodies with other parameters such as absolute CD4+ T cell count, serum p24 levels, etc., may assist in forming an improved prognostic indication for state of HIV infection.

Recent work: To strengthen our work in refining these epitopes, we sought to use recombinant peptides which extended the sequences derived from our preliminary data. We established a collaboration with Dr. Sean Nowlan, who had described three highly immunogenic regions of pol (15):

Epitope designation	Amino acid residues	HIV-1 nucleotides	Provisional epitope map
rI	9-214	2101-2774	144-191
rII	191-335	2700-3134	214-335
rIII	335-567	3134-3832	511-536

These pol sequences, and 5 additional non-reactive recombinant peptides from pol (15) which we have also received, will be valuable in refining the exact specificity of pol targets for both antibody and CTL.

III. RELATED PROJECT: Regulation of chronic HIV infection and its relationship to immune activation and immune modulators.

As is apparent from our list of WRAIR-assisted publications, while this work was in progress we came to realize

the importance of several biologic properties of HIV also relevant to immune intervention. HIV infection of CD4+ T lymphocytes and monocytes may lead to a low level chronic or latent phase during which there is little or no viral replication. Transition to a productive state can be triggered, at least in vitro, by immunologic activation of infected cells (16). These signals induce cellular trans-acting factors, which in turn stimulate transcrip- tion from the HIV long terminal repeat (LTR). The virus is then able to synthesize its own regulatory factors which further stimulate transcrip- tion from the LTR, leading to viral replication and cell death (17).

We explored phorbol ester-mediated rescue of virus from a clone of promonocyte cells, U1.1A, chronically infected with HIV-1. Cellular responses to phorbol esters mimic responses to growth factors, hormones, and immunologic activation. In terms of HIV induction via PMA or immunologic stimuli (antigen, alloantigen, lymphokine), PKC activation presumably initiates an intracellular phosphorylation cascade, the end result of which is to stimulate pre-existing nuclear trans-acting factor (18). NF-kB is the enhancer protein first described as capable of upregulating HIV replication by interacting with the 5' LTR at a site different from that of the tat-responsive TAR region (19). However, other PMA responsive sequences have been identified in viral sequences shared with HIV that affect other transcription factors (20). We reported (21) that PMA and PDB (4B-phorbol 12,13-dibutyrate) could lead to a productive infection not

elicited by congeners that bind to phorbol ester receptors but do not transduce an intracellular signal (Table II). Induction appeared specific to activation of PKC, and was blocked by three PKC inhibitors, H7, 2-aminopurine (Table III) and sangivamycin (data not shown). Its association with regulatory pathways involving the tat transcription unit was also explored. PMA-mediated enhancement of an HIV-LTR driven reporter gene was not blocked by H7 at concentrations capable of inhibiting HIV replication while the PMA effect was synergistic with tat-linked trans-activation.

There is the need to identify similar agents, with potential clinical utility, capable of blocking upregulation of virus in chronic HIV infection. Indeed, classic anti-virals such as 3'azido-3'deoxythymidine (AZT) have no effect in preventing induction of HIV expression in macrophage (U1.1) and other cell lines, while alpha-interferon may have a salutory effect (22).

As gonadal steriods and glucocorticoids can alter specific gene expression in at least one immunosuppressive murine retrovirus, mammary tumor virus (MMTV), we first explored the ability of dexamethasone (DXM) to upregulate latent HIV replication or to alter transcription at the HIV-1 long terminal repeat (LTR). Chronically infected lymphocytes as well as lymphocyte and monocyte (U1.1) cell lines were unperturbed by DXM used over braod concentrations (10^{-4} to 10^{-9} M) and time intervals (24-96 hrs.) (23). This unresponsiveness corresponded to the lack of a positive effect of DXM on HIV associated trans-

Table II. Effect of phorbol esters on induction of HIV-1 from chronically infected U1.1A promonocytic cells

Phorbol Ester	Concentration (ng/ml)	HIV-1 p24 core antigen ($pg/10^4$ cells)
None	-	2920
PMA	5	13,040 ± 1240
PMA	50	32,440 ± 4920
PMA	500	11,200 ± 600
PDB	5	31,880 ± 4920
PDB	50	59,400 ± 6320
PDB	500	46,840 ± 2800
phorbol 13-acetate	5	2480
phorbol 13-acetate	50	3600
phorbol 13-acetate	500	1040
4B-phorbol	5	4640
4B-phorbol	50	5040
4B-phorbol	500	3800

Table III. Effect of protein kinase C (PKC) inhibitors on induction of HIV-1 from chronically infected U1.1A promonocytic cells*

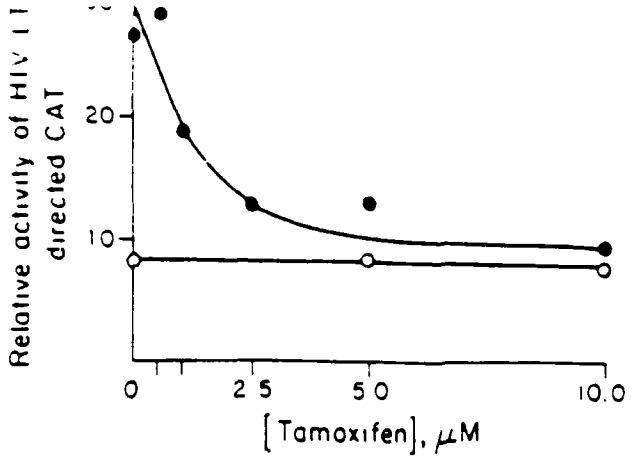
Expt.	PMA (5ng/ml)	PKC Inhibitor Agent	PKC Inhibitor Concentration (μM)	HIV-1 activity [p24] core Ag (pg/104 cells)	HIV-1 activity Inhibition (%)
A	-	-	-	1,880	-
	+	-	-	14,660 ± 1080	-
	+	H7	10	13,540 ± 650	7.6
	+	H7	50	5,840 ± 2590	60.2
	+	H7	75	2,150 ± 730	85.3
	+	H7	100	1,070	92.7
B	-	-	-	1,080	-
	+	-	-	13,770	-
	+	2-AP	10	17,930	0
	+	2-AP	50	2,890	79.0
	+	2-AP	75	710	94.8
	+	2-AP	100	640	95.4

*Cells were plated at 1 x 10^4/microwell in 0.2ml of culture medium together with buffer or PKC inhibitor (either the isoquinoline H7 or 2-aminopurine) for 1h at 37°C, followed by addition of the phorbol ester PMA. Culture supernatants were harvested 48h later and HIV p24 core antigen concentrations assessed by ELISA.

activation. These cells possessed the appropriate steriod receptors, as DXM downregulated Fc gamma type I receptors in both normal and HIV-infected monocytic cells. In addition, DXM could block the transcriptional enhancement of an HIV-LTR linked reporter gene by phorbol ester, while leaving basal levels of HIV-LTR directed transcription unperturbed (23).

We subsequently showed that rescue of virus could be blocked by the triphenylethylene anti-estrogen tamoxifen (TMX), at concentrations which did not affect cellular DNA synthetic responses. This effect correlated with tamoxifen's ability to completely inhibit PMA enhancement of HIV-promoter mediated trans-activation in both monocytic and CD4+ T cells (Fig. 1). No interference with a primary infection was noted. Tamoxifen's mechanism of action may relate both to its capacity to inhibit PKC, as well as to consensus sequences for gonadal steroid responsive elelments in the HIV LTR. Thus, TMX inhibited HIV-LTR-directed tat activity with an IC_{50} of 2uM, and its IC_{50} for inhibition of PKC is 6.1 uM (24); typical serum levels of this drug in man are on the order of 2uM (25).

Manipulation of gonadal HREs in relationship both to viral regulation and immune modulation may be especially pertinent in HIV infection for several reasons. First, TMX has a variety of immunologic effects. In vitro modulation of human lymphocyte reactivity includes increases in pokeweed mitogen-driven immunoglobulin production, inhibition of CD8+ T suppressor cell but not CTL phenomena, and augmentation of natural killer cell



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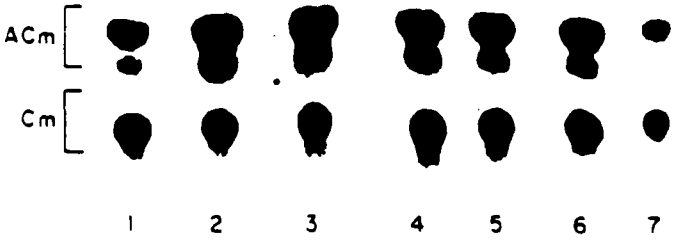

	1	2	3	4	5	6	7
Conversion (%)	16.9	57.8	62.0	39.0	26.0	28.1	19.7
PMA (50ng/ml)	-	+	+	+	+	+	+
TMX (μM)	0	0	0.5	1.0	2.5	5.0	10

Fig.1 . Effect of tamoxifen on PMA-mediated enhancement of HIV-LTR-CAT. The CAT plasmid (1μg DNA) was co-transfected into U1.1A cells (2 x 10^6) with a plasmid containing tat (1μg DNA). PMA (50 ng/ml) and/or TMX (1-10 uM) was present throughout the 48h culture period. The percent conversion of [^{14}C]chloramphenicol (Cm) to its acetylated forms (ACm) was determined.

A. Dose-response curve for inhibition of PMA-driven upregulation of HIV-LTR-CAT activity. This activity was measured in the presence (closed circles) and absence (open circles) of PMA.

B. Chromatographic data for conversion of Cm to ACm.

activity (26). Second, a prominent manifestation of HIV infection, Kaposi's sarcoma, is most prevalent among men. A model for this disease, produced when tat under the control of the HIV LTR was introduced into the germline of mice, was elicited only in male transgenic animals, despite equivalent levels of tat mRNA expression their female counterparts (27). The possibility that this sexual difference in phenotype is hormonally based is under investigation (27). A further indication that hormonal therapy might have a therapeutic role in HIV infection was the preliminary observation that the synthetic progesterone megestrol acetate (Megace) led to significant improvements in appetite, lethargy, and body weight in some HIV infected patients (28), and is under clinical evaluation at Walter Reed.

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